Detection of *Salmonella enteritidis*-Specific Immunoglobulin A Antibodies in Crop Samples from Chickens Infected with *Salmonella enteritidis*

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ABSTRACT The crop (ingluvies), an organ for food storage in most avian species when the proventriculus is full, is located at the base of the esophagus. Little is known about any immunological capacity in the crop, and the current study was conducted to determine whether any antibodies to SE could be found in crop flushes taken from White Leghorn hens following infection with this organism. Surprisingly, an exceptionally strong IgA anti-SE response could be detected in the crops of hens 17 d postchallenge, and a comparison at Day 22 of crop vs. intestinal IgA anti-SE responses showed a good correlation between anti-SE antibody levels in the two regions. Histologic examination of crop tissues revealed development of lymphoid aggregates in the crop walls following challenge with SE. These results indicate that the crop may serve a role in immune protection in addition to its capacity as a food storage organ.

(*Key words*: mucosal immunity, immunoglobulin A, *Salmonella*, crop, poultry immunity)

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INTRODUCTION

The crop (ingluvies) is an enlargement or out pouching of the esophagus proximal to the proventriculus or glandular stomach. Found in most avian species, the crop is generally regarded as a food storage area when the stomach is full (Dyce et al., 1996) and also moistens food with mucus to ease transit through the alimentary tract. Few other functions are attributed to this organ. However, the crop received significant attention recently as a source for *Salmonella* contamination of carcasses during processing (Chambers et al., 1998; Durant et al., 1999), which resulted in the crop being targeted as an important site for elimination of *Salmonella* from a food safety standpoint. This organ can become readily colonized with *Salmonella* following hen challenge (Barrow et al., 1988; Humphrey et al., 1993); however, little information is available regarding the presence of any immune response within the crop. During a recent study investigating mucosal immunity to SE in the alimentary tract of White Leghorn chickens, we discovered the presence of high titers of IgA specific for SE in samples extracted from crops removed from SE-infected, but not uninfected, birds. These samples were originally obtained by emulsifying crops in a stomacher-type apparatus. However, such extractions suffer from the possible contamination of samples with serous materials, and alternative methods of obtaining crop samples for assay were sought. The purpose of the current experiment is to describe the evolution of an immune response to SE in crops using material obtained by flushing the crop.

MATERIALS AND METHODS

Chickens

Thirty-two White Leghorn hens, 73 wk of age, were obtained from the specific-pathogen-free flock maintained at the Southeast Poultry Research Laboratory. The birds were housed in climate-controlled biocontainment buildings with full access to antibiotic-free feed and water. The presence of feed in the crop impedes the collection of secretions from this organ. Therefore, 12 h prior to killing, feed was removed to expedite the flushing protocols.

Infection and Flush Protocol

Five days prior to SE challenge, eight hens were killed via CO_2 inhalation. The crops were excised, and 10 mL of warm crop flush solution (1 *M* tris/glycine buffer with 0.25% Tween 20, pH 7 to 8 developed in our laboratory for obtaining intestinal flush samples) was injected into the crop lumen with a 10-cc syringe and an 18-ga needle. The selection criteria for the Tris-glycine buffer as the flush solution stems from earlier work, which demonstrated the utility of using glycine solutions in

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enteral perfusion studies (Iijima et al., 1997). The solution was aspirated and injected several times to flush the secretions from the crop wall, and then the contents were collected into the syringe and dispelled into a tube. The crop flushes were centrifuged for 5 min at 12,000 × *g*, and the supernatants from each sample were frozen at –20 C until assay. Freezing the samples ensured the prolonged viability of the immunoglobulins present in the fluids. The remainder of the birds were orally challenged with 1 mL of a 10^{-3} dilution of an overnight broth culture of a nalidixic acid-resistant SE (approximately 1×10^6 SE/mL). An additional eight birds were killed on Days 12, 17, and 24 postchallenge. The crops were excised and processed as above. For the birds killed on Day 22 postchallenge, the small intestine (duodenum to the ileal cecal junction) were also removed and flushed with 10 mL warm lavage solution, and the flush was processed as above for the crop.

IgA Assay

Crop and intestine samples were thawed and diluted 1:5 in PBS containing 0.05% Tween 20. The samples were added in duplicate to ELISA trays to which 10 μg/mL SE lipopolysaccharide (LPS)[2] had been adsorbed. Following room temperature incubation for 60 min, the plates were washed three times and then assayed for IgA anti-SE antibodies as described previously (Holt et al., 1999).

Histologic Examination of Tissue

Following killing, crops were removed from prechallenge and 3 wk postchallenge hens and placed into 10% buffered formalin. The tissues were processed, paraffin embedded, and serial cuts were made at 7.0 μm and placed on slides. The slides were stained using hematoxylin and eosin and examined by light microscopy.

Statistics

Differences between mean optical density 405 readings of ELISA results from the different weeks were examined via one-way ANOVA with significance expressed at $P < 0.05$ (Shott, 1990). Correlations between intestinal and crop ELISA optical density 405 readings were calculated using the Pearson correlation coefficient (Shott, 1990).

RESULTS AND DISCUSSION

The crop has received substantial attention as a region that becomes colonized by *Salmonella* (Barrow et al., 1988; Humphrey et al., 1993) and as a source of carcass contamination by food-poisoning organisms (Chambers et al., 1998; Durant et al., 1999). However, there is a paucity of information regarding the development of immunity in this organ in response to antigenic stimulation. Arai et al. (1988) found immunoglobulin-positive cells in the esophagus and proventriculus of chickens and Matsumoto and Hashimoto (2000) detected B- and T-cell populations in the chicken proventriculus. However, Vervelde and Jeurissen (1993) could detect no B cells in either region but did identify cell populations expressing T-cell and macrophage markers in these areas. These results do indicate a possible capacity for immunity in this region, although the picture is not clear especially for the crop. The current study examined the specific humoral response in the crops of chickens following challenge with an avian pathogen.

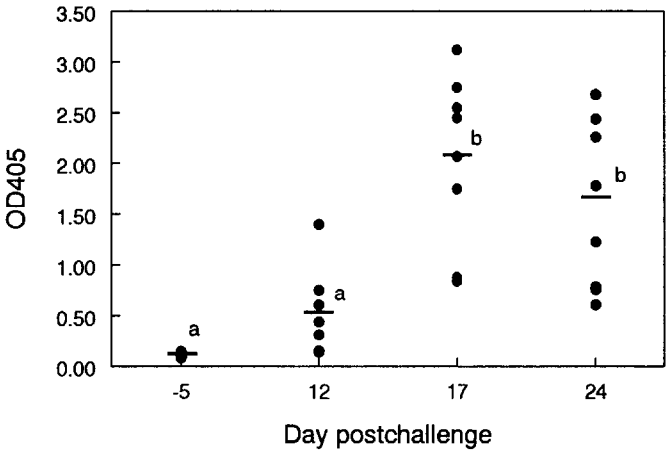

FIGURE 1. Change in IgA anti-SE levels over time in crops of chickens challenged with SE. Flushes from crops of chickens killed at various times pre- and postchallenge (eight chickens per time point) were assayed via an IgA ELISA. Each circle represents the optical density at 405 nm (OD405) reading from an individual bird and the line at each time period represents the mean OD405 reading for that group of eight birds. Means with different letters are significantly different ($P < 0.05$).

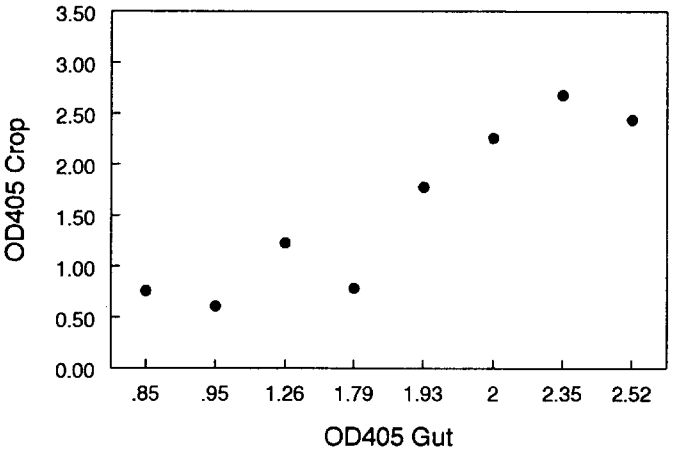

FIGURE 2. Plot of the IgA anti-SE levels in the crop vs. intestinal tract from chickens killed at Day 24 postchallenge. Flushes from crops and small intestine of chickens at Day 24 postchallenge were assayed via an IgA ELISA. Each circle represents the optical density at 405 nm (OD405) from the gut (X-axis) and the OD405 reading from the crop (Y-axis) from the same bird.

[2]Sigma Laboratories, St. Louis, MO.

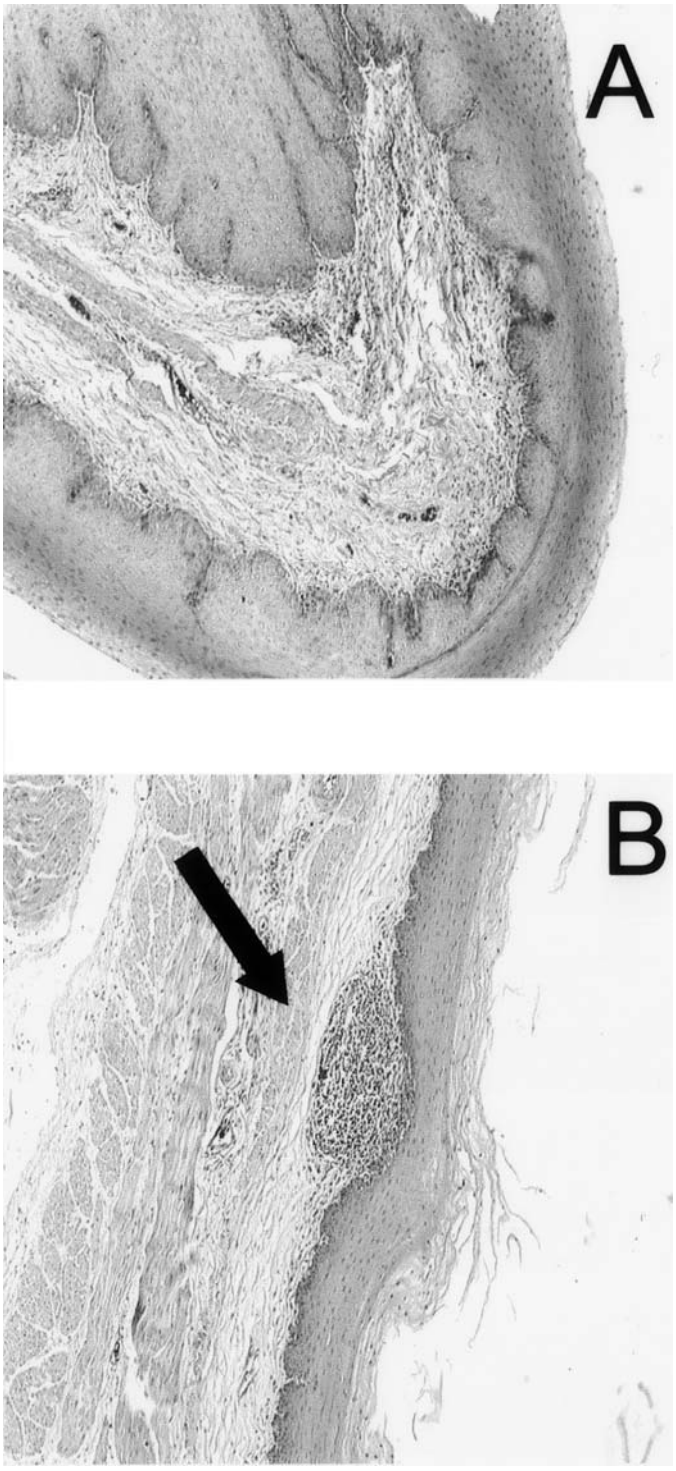

FIGURE 3. Histological examination of crop tissues from hens, pre-challenge (A) and 3 wk post-SE challenge. H & E stain at 10× magnification. Arrow points to lymphoid tissue aggregate.

Initial studies in our laboratory showed that SE flagellin-specific IgA can be detected from stomached crops removed from chickens infected with SE (Seo et al., 2002). The antigen used in the current study was SE LPS, which causes the problem of decreased assay specificity by detecting circulating antibodies from infections by other *Salmonella* serovars sharing the epitopes found in SE LPS. However, this antigen is much more readily available than SE flagella, and since the birds used in the experiments were obtained from the laboratory specific-pathogen-free flocks, prior exposure of these birds to *Salmonella* infections would be limited as would problems with LPS cross-reactivity. The levels of IgA to SE were minimal in the crop fluids obtained from the hens prior to challenge (Figure 1), but a strong response could be detected after the hens received the SE challenge. The bulk of the hens produced a detectable anti-SE response by Day 12 postchallenge. Higher responses and optical density readings averaging greater than 2.0 were observed at Day 17 ($P < 0.005$), and the responses remained high at Day 22 ($P < 0.01$). Along with crop samples, intestinal flush samples were also obtained on Day 22 and were assayed for SE-specific IgA levels. To determine how closely the crop responses reflect those of intestinal responses, a plot was formulated for each bird using the intestinal response as the y-coordinate and the bird's corresponding crop response as the x-coordinate. As shown in Figure 2, for most birds, a high correlation was found between crop vs. intestinal IgA levels—birds with high crop IgA readings generally had correspondingly high intestinal readings ($r = 0.88$). Although these results are preliminary, good correlations between IgA levels in the two regions indicate that crop IgA responses may be predictive of mucosal responses found in other regions of the alimentary tract. Immunoglobulin A was the only isotype tested in the current study, and the relative contribution of the IgG and IgM isotypes to the response also need to be evaluated.

The question arises whether antibodies found in the crop were received passively from other regions or synthesized locally in the crop mucosa. Esophageal tonsils, a well-developed aggregate of lymphoid tissue anterior to the crop (Arai et al., 1988) could serve as a source of the antibodies measured in the present study. Alternatively, reflux is a well-known phenomenon in poultry (Duke 1986), and the antibody source may be from the proventriculus (Matsumoto and Hashimoto, 2000) or from bile (Schat and Meyers, 1991). In the current study, lymphoid aggregates can be observed in the crop mucosa 3 wk postchallenge with SE (Figure 3B, arrow) compared with diffuse lymphoid tissue found in the crops of chickens prior to SE exposure (Figure 3A). The anti-SE antibodies may, therefore, result from an active immune response found in the crop or a combination of both passively acquired and actively synthesized antibodies.

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